ORIGINAL ARTICLE BACTERIOLOGY

Mycobacterium avium ssp. *hominissuis* biofilm is composed of distinct phenotypes and influenced by the presence of antimicrobials

M. McNabe[1], R. Tennant[1], L. Danelishvili[1], L. Young[2] and L. E. Bermudez[1,2,3]

1) *Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, OR,* 2) *Kuzell Institute, CPMCRI, San Francisco, CA and* 3) *Department of Microbiology, College of Science, Oregon State University, Corvallis, OR, USA*

Abstract

Mycobacterium avium ssp. *hominissuis*, hereafter referred to as *M. avium*, forms biofilm, a property that, in mice, is associated with lung infection via aerosol. As *M. avium* might co-inhabit the respiratory tract with other pathogens, treatment of the co-pathogen-associated infections, such as in bronchiectasis, would expose *M. avium* to therapeutic compounds that may have their origin in other organisms sharing the natural environments. Incubation of *M. avium* with two compounds produced by environmental organisms, streptomycin and tetracycline, *in vitro* at subinhibitory concentrations increased biofilm formation in a number of *M. avium* strains, although exposure to ampicillin, moxifloxacin, rifampin and trimethoprim–sulphamethoxazole had no effect on biofilm formation. No selection of genotypically resistant clones was observed. Although incubation of bacteria in the presence of streptomycin upregulates the expression of biofilm-associated genes, the response to the antibiotics had no association with the expression of a regulator (LysR) linked to the formation of biofilm in *M. avium*. Biofilms are composed of planktonic and sessile bacteria. Whereas planktonic *M. avium* is susceptible to clarithromycin and ethambutol (clinically used antimicrobials), sessile bacteria are at least three-fold to four-fold more resistant to antibiotics. The sessile phenotype, however, is reversible, and no selection of resistant clones was observed. Mice infected through the airway with both phenotypes were infected with a similar number of bacteria, demonstrating no phenotype advantage. *M. avium* biofilm formation is enhanced by commonly used compounds and, in the sessile bacterial phenotype, is resistant to clarithromycin and ethambutol, in a reversible manner.

Keywords: Antibiotics, biofilm, gene expression, *M. avium*
Original Submission: 5 March 2010; **Revised Submission:** 16 June 2010; **Accepted:** 1 July 2010
Editor: M. Drancourt
Article published online: 15 July 2010
Clin Microbiol Infect 2011; **17:** 697–703
10.1111/j.1469-0691.2010.03307.x

Corresponding author: L. E. Bermudez, Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University Corvallis, Corvallis, OR 97331, USA
E-mail: luiz.bermudez@oregonstate.edu

Introduction

Biofilm formation is an approach used by a number of organisms to survive under conditions of nutrient depletion [1]. *Mycobacterium avium* ssp. *hominissuis*, hereafter referred to as *M. avium*, is an environmental bacterium that commonly causes infection in individuals with chronic lung infection and AIDS [2–4]. One of the characteristics of *M. avium* is the ability to form biofilm in the environment [5]. Several genes have been linked with the ability to develop biofilm on both poly(vinyl carbonate) (PVC) and plastic surfaces. *M. avium* mutants deficient in those genes are impaired in biofilm formation *in vitro* [6]. More recently, it has been shown that *M. avium* lung infection in mice appears to be greatly dependent on the ability to colonize and establish biofilm on the surface of bronchial cells [6]. This finding offers, at least partially, a possible explanation for the difficulty in treating *M. avium* lung infection [7,8], which is frequently unsuccessful [9].

Although the presence of biofilm could be the explanation for many of the clinical aspects of *M. avium* complex lung infection, other factors may participate in and influence the formation and maintenance of biofilm, and the response to therapy. *M. avium* is an environmental organism that exists in the presence of other bacteria, both in the environment outside the host and in the host. For example, in both soil and water, *M. avium* shares the habitat with many other organisms, potentially forming complex colonies. In individuals with chronic lung disease, many pathogens, such as *Pseudomonas aeruginosa*, *Burk-*

holderia cepacia and *Staphylococcus aureus*, can be found co-habiting in the airways. It is common for these patients to have many episodes of infection, for which antibiotics are often prescribed. There is a possibility that some of the environmental microorganisms that produce natural antibacterial molecules used in therapy for humans share the soil with *M. avium*. Assuming that *M. avium* will have been in contact with some of the same antibacterial molecules before, it is likely that the bacterium will has developed defence mechanisms, which can certainly be triggered by the presence of the same or similar antimicrobial molecules found in the environment.

The biofilm structure is composed of at least two populations of microorganisms, planktonic and sessile. Sessile bacteria form the biofilm, and planktonic organisms occasionally detach (in both the pre-biofilm phase and the post-biofilm phase). A number of studies investigating a large number of pathogens, including *Mycobacterium abscessus*, have established that planktonic and sessile subpopulations have different susceptibilities to antibiotics [10]. Biofilm resistance to antibiotics has been attributed to at least two main factors. One is that biofilm creates a barrier against the penetration of antibiotics; and the other is that bacteria in the biofilm are not in the replicating state, and are therefore resistant to the majority of antibiotics.

In this study, we first examined whether several antibiotics that are commonly used to treat infections in patients with cystic fibrosis and other chronic lung diseases have any effect on the ability of *M. avium* to form biofilm. In addition, we evaluated the susceptibility of planktonic and sessile subpopulations to antibiotics that are frequently used to treat lung *M. avium* infection.

Materials and Methods

Bacteria

M. avium strains 101, 104 and A5 (kindly provided by K. Eisenach, University of Arkansas, Little Rock, USA) were used in the reported experiments. They were isolated from the blood of an AIDS patients. Strains 3362-33 and 3362-34 are *M. avium* strains isolated from the lungs of individuals with pulmonary pathology. They were kindly provided by R. Wallace (Tyler, TX, USA). Bacteria were grown on Middlebrook 7H10 agar supplemented with oleic acid, albumin, dextrose and catalase for 10 days at 37°C, harvested and used to establish a suspension of 5×10^8 CFU/mL. The suspension was then used to seed a PVC 96-well plate, as described previously [5,11,12]. The plate was kept at room temperature for 14 days. After this period, biofilm had developed on the surface of the walls. The biofilm-impaired strains 5G4 (in which MAV_3209 hypothetical membrane protein has been inactivated) and 6H9 (in which SucA has been inactivated) were cultured in agar plates in the presence of 200 mg/L kanamycin. The strains have been previously described [6,12] and are transposon mutants of strain A5. *M. avium* clone 8G12 was obtained by screening a transposon library for clones associated with increased ability to form biofilm on PVC. Clone 8G12 was selected because it has an eight-fold greater ability to form biofilm (relative increase as compared with the parent strain A5) than the wild-type strain A5. Sequencing of the inactivated gene determined that it is *MAV_2151*, which encodes a transcription regulator of the LysR family (data not shown).

Antibiotics

Streptomycin and tetracycline were initially isolated from environmental fungi and bacteria. Ampicillin, rifampin, moxifloxacin and trimethroprim–sulphamethoxazole were also used. All of the compounds, with one exception, were purchased from Sigma Chemicals (St Louis, MO, USA). Moxifloxacin was obtained from Bayer (West Haven, CT, USA). Different concentrations were obtained by dilution in Hank's balanced salt solution (HBSS). Clarithromycin was provided by Abbott Laboratories (Chicago, IL, USA) and ethambutol was purchased from Sigma.

Antibiotic susceptibility testing was performed with both planktonic and sessile phenotypes, as previously described, with a broth microdilution method [11]. Briefly, 1×10^5 bacteria were seeded in 0.3 mL of 7H9 Middlebrook broth with oleic acid, albumin, dextrose and catalase in a 96-well microtitre plate (Falcon, VWR, West Chester, PA, USA). The number of bacteria for the inoculum was established by using a turbidity method, and confirmed by plating onto 7H10 agar. The concentrations of clarithromycin and ethambutol used ranged from 0.125 to 128 mg/L.

Biofilm assays and phenotypes

For the assays, we used three strains of *M. avium* isolated from blood of AIDS patients (101, 104, and A5) and two strains isolated from lung infection (3362-33 and 3362-34). Bacteria were grown on Middlebrook 7H10 agar plates, and 1×10^7 bacteria in 0.3 mL of HBSS were then seeded in wells of 96-well PVC plates. Two hours after seeding, bacteria were exposed to either tetracycline or streptomycin at 0.5, 1 or 2 mg/L (MICs >128 and 8 mg/L, respectively), ampicillin 10 mg/L, moxifloxacin 1 mg/L, trimethoprim–sulphamethoxazole 3 mg/L or rifampin 1 mg/L (subinhibitory concentrations). Antibiotics were added again at day 7, and PVC plates were followed for 14 days. Biofilm formation was then quantified as previously reported [5,12], by measuring

the amount of biofilm by staining it with crystal violet and using the intensity of the staining to determine the exact biofilm mass, by means of spectrophotometry.

To determine whether the phenotype was temporary or permanent, sessile bacteria were passed on 7H10 agar plates twice, and the MICs of both clarithromycin and ethambutol were determined.

To obtain both subpopulations, PVC plates with *M. avium* biofilm were allowed to remain at rest for 14 days. Then, the supernatant was removed and centrifuged at 4000 ***g*** for 20 min at 4°C to collect the bacterial pellet. The pellet, composed of planktonic bacteria, was washed at 4°C with HBSS, passed through a 24-gauge needle ten times and resuspended in fresh HBSS. The concentration was adjusted to 5×10^7 CFU/mL. The biofilm, composed of a sessile population of bacteria, was then removed with a cotton swab and resuspended in HBSS at 4°C. The bacteria were spun down as described above, and then passed through a 24-gauge needle ten times to disperse them in the suspension; the concentration was then adjusted to 5×10^7 CFU/mL. Both subpopulations were maintained at 4°C for up to 3 days before the assays, in order to conserve the phenotype. Bacteria were then placed in 7H9 broth suspension with different concentrations of antibiotics, and plated for quantification after 48 h. The bacterial concentration in the inoculum was determined by plating the suspensions onto 7H10 agar.

To determine whether clones of planktonic and sessile bacteria were resistant to 20 mg/L clarithromycin or ethambutol, bacterial suspensions of 10^8 CFU/mL were plated onto 7H10 agar containing either clarithromycin or ethambutol. The same inoculum was plated onto 7H10 agar without antibiotics. Colonies obtained from plates with antibiotics were additionally tested individually for susceptibility, as described above.

Mouse experiments

Female C57BL/6 mice, weighing 20 g, were purchased from Jackson Laboratory (West Grove, PA, USA). After 1 week of acclimation, mice were infected with 10^8 bacteria delivered to the nostrils in 0.2 mL of HBSS. Twelve mice were used per time-point. Mice were harvested at day 7 and at 5 weeks following infection. Lungs were removed, homogenized, diluted and plated onto 7H11 agar containing trimethoprim–sulphamethoxazole, polymyxin B, carbenicillin and amphotericin B, as previously reported [6]. Quantification of CFUs was performed after 14 days.

Biofilm-related genes

As exposure to streptomycin and tetracycline resulted in increased biofilm formation, it was important to determine whether the antibiotics triggered the expression of biofilm-related genes. Both *guaB2* and *gtf* were selected [12]. Those genes are involved in glycopeptolipid synthesis and synthesis of lipoarabinomannan, respectively. Strain 104 was exposed to 1 mg/L streptomycin or 1 mg/L tetracycline for 2 h, and RNA purification and cDNA synthesis were performed as previously described [13]. The cDNA was amplified using the following primers: GuaB2 F, 5′-TCA CCT GCC GCC CCG ACA ACA CGC TGC CCC-3′; GuaB2 R, 5′-GGC ACC CGG CCC TCG ATG CCC TCG GGC ACC-3′; Gtf F, 5′-ATG GAG GGC GCC GAC GTG CCC-3′; and Gtf R, 5′-AGG ATC GCG GTG ATG CTG CCC-3′.

Real-time PCR was carried out as previously described [12]. Briefly, quantitative fluorogenic amplification of cDNA was performed using the iCycler (Bio-Rad) and SYBR green technology (Bio-Rad, Hercules, CA, USA), according to the method previously described [12].

Statistical analysis

Experiments were repeated at least three times, and the results are expressed as a mean ± standard deviation. The comparisons among experimental groups were performed with ANOVA and Student's *t*-test when appropriate. A p-value of <0.05 was considered to be statistically significant.

Results

Antibiotics and biofilm

It was observed that exposure to streptomycin at subinhibitory concentrations resulted in increased *M. avium* biofilm formation. In fact, incubation in the presence of antimicrobial molecules produced by environmental microorganisms may impact on the formation of biofilm by other environmental bacteria. AIDS-derived *M. avium* strains placed in contact with tetracycline were induced to produce significantly more biofilm, although the significance of the increase in biofilm formation related to tetracycline exposure may be understated in relation to the MIC of tetracycline and the concentration of the antibiotic that *M. avium* was exposed to. Incubation with streptomycin at 0.5 mg/L also induced AIDS-derived *M. avium* to produce increased amounts of biofilm. When pulmonary strains of *M. avium* were exposed to either tetracycline or streptomycin at all tested concentrations, both strains showed significant increases in biofilm production (Tables 1 and 2). Exposure to ampicillin, moxifloxacin or trimethoprim–sulphamethoxazole did not result in any change in biofilm formation (Table 3). The use of rifampin was associated with a decrease in the biofilm mass of 10% ± 6% ($p > 0.05$). When mutants impaired in biofilm formation—6H9 and 5G4—and 8G12, a mutant that forms

TABLE 1. Effect of streptomycin exposure on biofilm formation by *Mycobacterium avium* strains

	Biofilm formation (OD ± SD)[a]			
		Streptomycin (mg/L)[b]		
M. avium strain	No antibiotic	0.5	1	2
104	0.236 ± 0.026	0.156 ± 0.032[c]	0.534 ± 0.046[c]	0.549 ± 0.041[c]
101	0.243 ± 0.029	0.584 ± 0.041[c]	0.563 ± 0.041[c]	0.545 ± 0.065[c]
A5	0.266 ± 0.041	0.668 ± 0.036[c]	0.681 ± 0.044[c]	0.685 ± 0.048[c]
3362-33	0.222 ± 0.032	0.508 ± 0.016[c]	0.628 ± 0.023[c]	0.699 ± 0.037[c]
3362-34	0.278 ± 0.048	0.497 ± 0.039[c]	0.548 ± 0.062[c]	0.591 ± 0.056[c]
A5	0.286 ± 0.045	0.647 ± 0.051[d]	0.670 ± 0.041[d]	
8G12	0.592 ± 0.051	0.732 ± 0.056[e]	0.744 ± 0.026[e]	
5G4	0.159 ± 0.040[a]	0.194 ± 0.024[e]	0.211 ± 0.034[e]	
6H9	0.123 ± 0.067[a]	0.143 ± 0.042[e]	0.173 ± 0.028[e]	

OD, optical density; SD, standard deviation.
[a]Biofilm mass was measured by staining the biofilm with crystal violet and determining the OD of the crystal violet by spectrophotometry [5,11].
[b]The results are the means of three independent experiments.
[c]$p < 0.05$ as compared with biofilm without exposure to antibiotic.
[d]$p < 0.05$ as compared with A5 without exposure to antibiotic.
[e]$p > 0.05$ as compared with the mutant without exposure to antibiotic.

TABLE 2. Effect of tetracycline exposure on biofilm formation by *Mycobacterium avium* strains

	Biofilm formation (OD ± SD)[a]			
		Tetracycline (mg/L)[b]		
M. avium strains	No antibiotic	0.5	1	2
104	0.256 ± 0.026	0.310 ± 0.042	0.316 ± 0.031[c]	0.350 ± 0.017[c]
101	0.243 ± 0.029	0.314 ± 0.046	0.327 ± 0.023[c]	0.329 ± 0.036[c]
A5	0.266 ± 0.041	0.312 ± 0.022	0.336 ± 0.039[c]	0.342 ± 0.028[c]
3362-33	0.222 ± 0.032	0.371 ± 0.033[c]	0.384 ± 0.040[c]	0.388 ± 0.019[c]
3362-34	0.206 ± 0.057	0.423 ± 0.042[c]	0.457 ± 0.040[c]	0.481 ± 0.050[c]

OD, optical density; SD, standard deviation.
[a]The biofilm mass was measured by staining the biofilm with crystal violet and determining the OD of the crystal violet by spectrophotometry [5,11].
[b]The results represent three independent experiments.
[c]$p < 0.05$ as compared with biofilm not exposed to antibiotic.

an increased amount of biofilm as compared with the wild-type bacterium, were exposed to streptomycin and tetracycline (data not shown), the 6H9 and 5G4 mutants did not produce increased amounts of biofilm (Table 1). Exposure of the mutant that produces a high amount of biofilm to streptomycin resulted in a small, but consistent, increase in biofilm formation (Table 1).

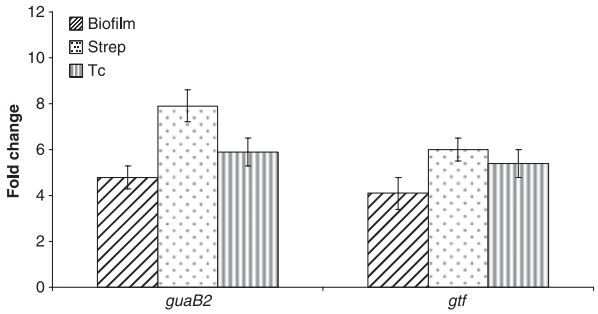

FIG. 1. Real-time PCR showing the fold increase, as compared with the bacteria not exposed to antibiotics, in the expression of *guaB2* and *gtf*. Biofilm, no antibiotic treatment; Strep, in the presence of 1 mg/L streptomycin; Tc, in the presence of 1 mg/L tetracycline. $p < 0.05$ for the comparison between biofilm and biofilm exposed to streptomycin. The assay was run as described in Materials and Methods.

Biofilm-related genes

In order to examine whether exposure to streptomycin or tetracycline induces biofilm-related gene expression in *M. avium*, 104 biofilm was exposed to 1 mg/L of each antibiotic, and the expression of *guaB2* and *gtf* was monitored. As shown in Fig. 1, in agreement with previously published data, both genes were expressed during biofilm formation. Gene expression was, however, significantly greater when antibiotics were applied to biofilms.

We established the MICs of clarithromycin and ethambutol for strains 104, 101, A5, 3362-33 and 3362-34, and the 5G4 and 6H9 mutants. All of the strains had an MIC of 2 mg/L for clarithromycin, except for 3362-33, for which the MIC was 4 mg/L. Similarly, the MICs of ethambutol did not vary significantly, with all of the strains, except for 101 and A5 (MIC 2 mg/L), being inhibited at 1 mg/L.

TABLE 3. Biofilm formation among *Mycobacterium avium* strains exposed to ampicillin, moxifloxacin, rifampin and trimethoprim–sulphamethoxazole (TMP-SMX)

	Biofilm formation (% increase/decrease) at 7 days				
M. avium strain	No antibiotic	Ampicillin (10 μg/L)	Moxifloxacin (1 μg/L)	TMP-SMX (3 μg/L)	Rifampin (1 μg/L)
104	0.271 ± 0.032	1 ± 0.3	0.1 ± 0.02	0.2 ± 0.03	−10 ± 6[a]
101	0.266 ± 0.049	0.4 ± 0.1	0.2 ± 0.02	0.3 ± 0.03	−7 ± 3[a]
A5	0.296 ± 0.057	0.3 ± 0.07	0.2 ± 0.05	0.2 ± 0.08	−9.2 ± 3[a]
3362-33	0.274 ± 0.037	0.2 ± 0.03	0.2 ± 0.1	0.2 ± 0.05	−10.1 ± 4[a]
3362-34	0.230 ± 0.025	0.1 ± 0.06	0.1 ± 0.09	0.2 ± 0.06	−9.6 ± 2[a]

[a]$p < 0.05$ as compared with biofilm formation without antibiotic exposure.
Results represent the mean percentage increase in biofilm formation of two different experiments.

TABLE 4. **MICs of clarithromycin and ethambutol for planktonic and sessile subpopulations of *Mycobacterium avium***

Subpopulation of *M. avium* 104	MIC (mg/L)	
	Clarithromycin	Ethambutol
Planktonic[a]	4	2
Sessile[a]	64[b]	16[b]
Planktonic[c] passed in medium	4	2
Sessile[c] passed in medium	4	2

[a]Planktonic and sessile subpopulations were collected as described in Materials and Methods. Bacteria were kept at 4°C to maintain the phenotype, and then placed in 7H9 broth with different concentrations of antibiotics for 48 h. The inoculum was determined by plating onto 7H10 agar without antibiotics.
[b]$p < 0.05$ as compared with the the planktonic subpopulation.
[c]Planktonic and sessile subpopulations were cultured twice on 7H10 agar at 37°C for 10 days before the determination of MIC.

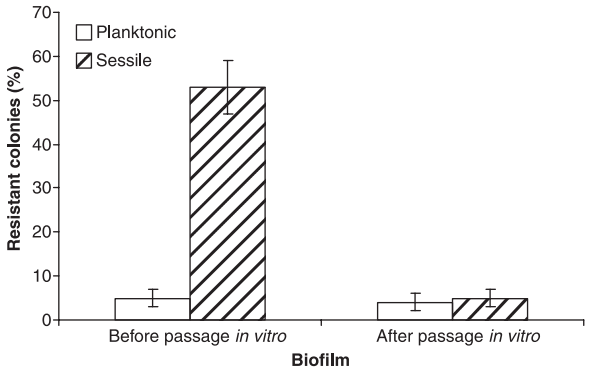

FIG. 2. Selection of resistant and susceptible bacteria. Planktonic and sessile subpopulations were plated onto 7H10 agar. The 7H10 agar plates contained 20 mg/L clarithromycin. The figure shows how many of the original colonies were resistant to antibiotics.

Planktonic and sessile subpopulations

To examine the MICs of the compounds for two subpopulations of *M. avium* in biofilm, planktonic and sessile, both populations were purified and bacteria were kept at 4°C until the assay. In addition, the two populations were cultured in 7H9 broth for two passages of 10 days before determination of MICs. Table 4 shows that sessile bacteria had MICs of clarithromycin five-fold greater than those of the planktonic bacteria, and an MIC of ethambutol four-fold greater than for the planktonic bacteria. Sessile bacteria passed twice in 7H9 broth underwent a reversion of the phenotype, and had MICs similar to those of planktonic *M. avium*.

To determine whether planktonic phenotype and the biofilm (sessile) phenotype would be able to infect mice equally, mice were exposed to bacteria (planktonic and sessile) via aerosol, and harvested at 7 days and 5 weeks after infection. The results (Table 5) show that the levels of infection with both strains were similar with regard to the number of CFUs in lungs and spleen, suggesting that both phenotypes are able to establish infection equally.

Sessile bacteria showed increased resistance to antibiotics. To investigate whether the phenotype is stable and represents the selection of a subpopulation of *M. avium*, sessile and planktonic clones were plated on 7H10 agar or 7H10 agar with clarithromycin at 20 mg/L, and the percentage of resistant colonies was quantified. As shown in Fig. 2, the resistant phenotype for clarithromycin was observed in the first passage, but was lost in subsequent passages.

Discussion

M. avium is an environmental pathogen that causes lung disease in patients with underlying pulmonary conditions [14]. *M. avium* is believed to be acquired primarily from the environment, most frequently from soil and water sources [15,16]. It has been demonstrated that the bacterium can be isolated from biofilms on sauna walls and in urban water systems [15,16]. More recently, it has been shown that *M. avium* can be commonly cultured in large amounts from household shower heads [17]. Therefore, individuals are exposed frequently to the bacterium, and those with risk factors may develop disease.

M. avium's ability to form biofilm appears to be necessary for it to cause pulmonary infection in mice. Mutant strains with deficiencies in developing biofilm had impaired ability to cause disease when delivered to the mice by aerosol [6].

TABLE 5. **Infection of C57BL/6 mice with planktonic and sessile *Mycobacterium avium* strain 104**

Bacteria[a]	CFU/lung and spleen			
	Spleen[b]		Lung[b]	
	Day 7	Week 5	Day 7	Week 5
Planktonic	$(6.1 \pm 1) \times 10^4$	$(3 \pm 2) \times 10^7$	$(2 \pm 0.9) \times 10^5$	$(7.1 \pm 1.8) \times 10^8$
Sessile	$(8.7 \pm 0.7) \times 10^4$	$(5.1 \pm 0.8) \times 10^7$	$(3.3 \pm 1.1) \times 10^5$	$(9.6 \pm 0.6) \times 10^8$

[a]Inoculum: planktonic, 1.4×10^8; sessile, 1.6×10^8.
[b]p-values were not significant between the experimental groups at all time-points.

Environmental bacteria are likely to compete with other bacteria for nutrients and survival. Many bacteria are known to produce antimicrobials that are lethal against other environmental organisms. Natural evolution would support the development of defence mechanisms, which often involve biofilm formation, in order to defend against environmental bacterium-generated products. The current opinion is that many of these products released into the environment work as signalling molecules for metabolic pathways in other organisms [18].

We show in this study that *M. avium* responds with an increase in biofilm formation when exposed to streptomycin and tetracycline at subinhibitory concentrations, but not when incubated with ampicillin, trimethoprim–sulphamethoxazole, moxifloxacin or rifampin. Tetracycline had a less accentuated effect against some of the strains tested. The finding was substantiated by the enhanced expression of *M. avium* biofilm-related genes upon exposure to antibiotics. Interestingly, ampicillin is derived from *Penicillium* and rifampin from *Nocardia*. The importance of the observation is that many patients with chronic lung conditions are treated with antibiotics, such as aminoglycosides or tetracyclines, for infections caused by many pathogens [19]. Therefore, there is a possibility that, in cases where *M. avium* is colonizing an individual receiving antibiotic, either for prophylaxis or for treatment, this could potentially result in the production of increased amounts of biofilm and further establishment of the infection.

In terms of existence and evolution, host infection is a consequence of *M. avium* survival in the environment. *M. avium* utilizes many of the features necessary to overcome environmental challenges and to infect and persist in the host. Among them are genes associated with amoebal invasion [20] and genes linked to biofilm formation [12]. Here we show that an iatrogenic action, antibiotic use, reproduces the environmental conditions of mixed biofilms, in which the bacterial structure becomes more resistant to damage inflicted from outside. Therefore, special care should be taken when a decision is made to administer antimicrobials to patients with chronic lung conditions in which the presence of *M. avium* is a possibility. Future studies will be needed to determine whether the effect of antibiotics *in vivo*-formed biofilm follows similar dynamics.

Once in a particular environment, biofilms are composed of at least two populations of bacteria, planktonic and sessile. The results obtained confirmed what many other groups working with different pathogens have already observed. The sessile phenotype is resistant to antibiotics, but the planktonic bacteria are susceptible. Such resistance has been described as being associated with the organization of the biofilm, but when biofilm was dispersed, bacteria still expressed a significant resistant phenotype. The mechanism for resistance (or reduced susceptibility) is unknown; however, the presence of biofilm matrix and its impact on bacterial phenotype, e.g. upregulation of bacterial antibiotic pump expression, could be factors influencing the phenotype.

Many studies in other systems have looked at planktonic phenotypes of pathogens. Recent work has evaluated the susceptibility of the planktonic population form of *M. abscessus* and determined that it is susceptible to antibiotics [10]. There is, however, no information on the detachment of planktonic bacteria from biofilms and the mechanisms of that detachment.

In summary, *M. avium* forms increasing amounts of biofilm in the presence of antibiotics such as streptomycin and tetracycline, which stimulate biofilm-related gene expression in the bacterium. Once formed, biofilms are composed of two distinct populations of bacteria: sessile, the more resistant phenotype; and planktonic, a susceptible phenotype. The role of clinically used antibiotics in stimulating signal transduction and metabolic pathways in bacteria is not well known, and should be studied further in mycobacteria.

Acknowledgements

We are indebted to D. Weber for help in editing and preparing the manuscript. We also thank C. Lambros for reviewing the manuscript, and N. Motamedi for technical help.

Funding

This work was supported in part by the National Institutes of Health (contract number AI-25468 to L. Young) and the National Institutes of Health (grant number AI043199 to L. E. Bermudez).

Transparency Declaration

No conflict of interest to declare.

References

1. Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G. The application of biofilm science to the study and control of chronic bacterial infections. *J Clin Invest* 2003; 112: 1466–1477.
2. Ebihara T, Sasaki H. Image in clinical medicine. Bronchiectasis with *Mycobacterium avium* complex infection. *N Engl J Med* 2002; 346: 1372–1378.

3. Fujita J, Ohtsuki Y, Suemitsu I *et al.* Pathological and radiological changes in resected lung specimens in *Mycobacterium avium intracellulare* complex disease. *Eur Respir J* 1999; 13: 535–540.
4. Martinez A, Torello S, Kolter R. Sliding motility in mycobacteria. *J Bacteriol* 1999; 181: 7331–7338.
5. Carter G, Wu M, Drummond DC, Bermudez LE. Characterization of biofilm formation by clinical isolates of *Mycobacterium avium*. *J Med Microbiol* 2003; 52: 747–752.
6. Yamazaki Y, Danelishvili L, Wu M *et al.* The ability to form biofilm influences *Mycobacterium avium* invasion and translocation of bronchial epithelial cells. *Cell Microbiol* 2006; 8: 806–814.
7. Griffith DE, Brown BA, Cegielski P, Murphy DT, Wallace RJ Jr. Early results (at 6 months) with intermittent clarithromycin-including regimens for lung disease due to *Mycobacterium avium* complex. *Clin Infect Dis* 2000; 30: 288–292.
8. Griffith DE, Brown BA, Murphy DT, Girard WM, Couch L, Wallace RJ Jr. Initial (6-month) results of three-times-weekly azithromycin in treatment regimens for *Mycobacterium avium* complex lung disease in human immunodeficiency virus-negative patients. *J Infect Dis* 1998; 178: 121–126.
9. Reich JM, Johnson RE. *Mycobacterium avium* complex pulmonary disease. Incidence, presentation, and response to therapy in a community setting. *Am Rev Respir Dis* 1991; 143: 1381–1385.
10. Greendyke R, Byrd TF. Differential antibiotic susceptibility of *Mycobacterium abscessus* variants in biofilms and macrophages compared to that of planktonic bacteria. *Antimicrob Agents Chemother* 2008; 52: 2019–2026.
11. Carter G, Young LS, Bermudez LE. A subinhibitory concentration of clarithromycin inhibits *Mycobacterium avium* biofilm formation. *Antimicrob Agents Chemother* 2004; 48: 4907–4910.
12. Yamazaki Y, Danelishvili L, Wu M, Macnab M, Bermudez LE. *Mycobacterium avium* genes associated with the ability to form a biofilm. *Appl Environ Microbiol* 2006; 72: 819–825.
13. Harriff MJ, Danelishvili L, Wu M *et al.* Mycobacterium avium genes mav_5138 and mav_3679 are transcriptional regulators that play a role in invasion of epithelial cells, in part by their regulation of cipa, a putative surface protein interacting with host cell signaling pathways. *J Bacteriol* 2009; 191: 1132–1142.
14. Aksamit TR. *Mycobacterium avium* complex pulmonary disease in patients with pre-existing lung disease. *Clin Chest Med* 2002; 23: 643–653.
15. Torvinen E, Lehtola MJ, Martikainen PJ, Miettinen IT. Survival of *Mycobacterium avium* in drinking water biofilms as affected by water flow velocity, availability of phosphorus, and temperature. *Appl Environ Microbiol* 2007; 73: 6201–6207.
16. von Reyn CF, Maslow JN, Barber TW, Falkinham JO 3rd, Arbeit RD. Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet* 1994; 343: 1137–1141.
17. Falkinham JO 3rd, Iseman MD, de Haas P, van Soolingen D. *Mycobacterium avium* in a shower linked to pulmonary disease. *J Water Health* 2008; 6: 209–213.
18. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 2005; 436: 1171–1175.
19. Chodosh S. Treatment of acute exacerbations of chronic bronchitis: state of the art. *Am J Med* 1991; 91: 87S–92S.
20. Danelishvili L, Wu M, Stang B *et al.* Identification of *Mycobacterium avium* pathogenicity island important for macrophage and amoeba infection. *Proc Natl Acad Sci USA* 2007; 104: 11038–11043.